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Bioactive xanthenes from the roots of *Hypericum perforatum* (common St John's wort)

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Abstract

BACKGROUND—Extracts of *Hypericum perforatum* L. (common St John's wort; Hypericaceae) are sold as phytopharmaceuticals and herbal supplements to treat mild to moderate depression and as food additives. Extensively cultivated in Europe, plants can be infected by anthracnose (*Colletotrichum gloeosporioides*), a virulent fungal pathogen that causes tissue necrosis and dramatically decreases crop value. Such infections triggered the production of new secondary metabolites, specifically xanthenes, in cell culture experiments.

RESULTS—Bioassay-guided fractionation of *H. perforatum* root extracts, testing for growth inhibition of plant pathogenic fungi from the genera *Colletotrichum*, *Botrytis*, *Fusarium* and *Phomopsis*, was performed. *In vitro* anti-inflammatory activity through inhibition of COX-1, COX-2 and 5-LOX-catalyzed LTB₄ formation was also evaluated. Extracts were analyzed by various chromatographic means and structure elucidation was performed using data from nuclear magnetic resonance and mass spectrometry.

CONCLUSION—Researchers have previously described constituents from the aerial parts of this species, but few reports describe secondary metabolites found in underground parts, of particular interest because the lower stem and upper root are often sites of fungal infection. This work resulted in the isolation of three xanthenes: 1,6-dihydroxy-5-methoxy-4',5'-dihydro-4',4',5'-trimethylfurano-(2',3':3,4)-xanthone; 4,6-dihydroxy-2,3-dimethoxyxanthone; and *cis*-kielcorin, one of which possessed novel bioactivity against species of *Phomopsis* and inhibited 5-LOX-mediated LTB₄ formation.

Keywords

Hypericaceae; *Colletotrichum*; anthracnose; *Phomopsis*; xanthenes; anti-inflammatory

INTRODUCTION

Hypericum perforatum L. is a perennial herb that is found in its native range distributed throughout Eurasia and which has been incorporated into folklore and is used as traditional

medicine in that part of the world. The inflorescences of *H. perforatum* are traditionally prepared as a decoction or infusion and taken orally for sedative and tonic purposes, or applied externally as a poultice or prepared as an oil infusion to treat sciatica, neuralgia and to speed wound healing.¹ Several classes of bioactive secondary metabolites, including naphthodianthrone (e.g., hypericin and pseudohypericin), flavonol glycosides (e.g., isoquercitrin and hyperoside), biflavonoids (e.g., amentoflavone), phloroglucinol derivatives (e.g., hyperforin, adhyperforin) and xanthones have been isolated from the aerial parts of *H. perforatum*.² Although compounds isolated from *H. perforatum* have displayed significant activity in many bioassays, much recent research has focused upon antidepressant and anti-inflammatory activity.

Owing to the usage of *Hypericum* extracts in contemporary alternative medicine, its cultivation and production have increased and expanded to multiple continents during the past 20 years.³ As one of the most important medicinal plants used in western herbal medicine, *H. perforatum* was among the top 10 best-selling herbal dietary supplements in the USA, with sales in 2008 estimated at around US \$8.2 million.⁴ In 2004, *H. perforatum* represented nearly 13% of all European herbal market sales, which were valued at more than €70 million in Germany alone.⁵ For this reason, pathogens targeting *H. perforatum* and causing a reduction in either biomass or product quality are of significant interest. One agronomic strategy to combat such losses is to identify secondary metabolites naturally occurring in individuals or populations of *H. perforatum*, which may provide resistance to infection by these pathogens, and subsequently to focus energies upon selective breeding of plants with desired biosynthetic capabilities.³

The plant pathogenic fungus *Colletotrichum gloeosporioides* Penz., which causes a disease known as anthracnose, has been identified as a pathogen of *H. perforatum*.³ Filamentous fungi of the genus *Colletotrichum* and its teleomorph *Glomerella* are major pathogens worldwide. *Colletotrichum* species often cause the typical symptoms of sunken necrotic lesions, usually with red margins.^{6,7} Research aimed at the development of new methods to control anthracnose of strawberry fruits showed that naturally occurring fungitoxic compounds vary between anthracnose-susceptible and anthracnose-resistant cultivars. These metabolites are also present in different amounts in vegetative tissues of different ages.⁸ Additional plant pathogenic fungi of interest in our laboratories include members of the genera *Botrytis*, *Fusarium* and *Phomopsis*. *Phomopsis* cane and leaf spot (*P. viticola* Sacc.) and *Botrytis* bunch rot (*B. cinerea* Pers.) cause serious economic losses to the vine grape industry in the USA and Europe, while *P. obscurans* (Ell. & Ev.) Harter causes *Phomopsis* leaf blight and fruit rot of strawberry. *Fusarium oxysporum* Schltdl. (Panama disease or Agent Green) negatively affects numerous crop plants in the western hemisphere, and has been reported as an occasional plant pathogen of *H. perforatum*.^{9,10}

In *H. perforatum*, symptoms of *Colletotrichum* infection include increased production of anthocyanidins: flavonoid structures that are formed through biosynthetic contributions from both the shikimate and polyketide pathways, leading to a red- to purple-brown coloration in above-ground tissues. Many studies have positively correlated anthocyanidin production with exposure to environmental stress factors and researchers hypothesize that these compounds play a role in the plant adaptive defense response. Necrotic stem lesions, which can girdle the stem base in *H. perforatum*, appear as the *Colletotrichum* infection worsens.³ Interestingly, the physical phenomenon of stem girdling in maples (*Acer*) has also been shown to significantly increase anthocyanidin expression, resulting in higher proportions of red to purple-colored foliage, indicating that the co-occurrence of these symptoms is neither limited to a particular plant family nor directly resulting from infection with this particular fungus.¹¹ The leaves of *H. perforatum* on erect flowering stems adjacent to *Colletotrichum*-infected vegetative basal rosettes first become chlorotic, then turn red to brownish. Although

girdling lesions around the vegetative stems often prove fatal, functional flowers and fertile seeds are often produced by the adjacent flowering stems.¹²

Several *Colletotrichum* species have been shown to act as elicitors of biochemical plant defenses in plants. For example, *C. lindemuthianum* cell wall components were shown to induce the transcription of phenylalanine ammonia-lyase (PAL), an important enzyme that allocates carbon from phenylalanine into secondary metabolites stemming from the shikimate biosynthetic pathway, when alfalfa cell cultures were challenged.¹³ Vincent *et al.* showed that strawberry challenged with *C. fragariae* spores produced three phenolic defense-related compounds.⁸ In this study, anthracnose disease-resistant cultivars produced ~15 times more antifungal chemical constituents than did disease-susceptible cultivars. Meazza *et al.* reported on the antifungal activity of several classes of quinones and related compounds for activity and selectivity against *Colletotrichum acutatum*, *C. fragariae* and *C. gloeosporioides*.¹⁴

Alterations in the biosynthesis of phenolic constituents specifically in *Hypericum*, including an increase in xanthone production – compounds related to flavonoids that are also biosynthesized via the shikimate and polyketide pathways – have been observed following elicitation of cell suspension cultures of *H. perforatum* with cell wall extracts of *C. gloeosporioides*.¹⁵ In addition, both the biosynthesis of previously present xanthones and *de novo* production of new xanthones were boosted when cell suspension cultures of *H. perforatum* were elicited through co-cultivation with the Gram-negative, soil-borne plant pathogenic bacterium *Agrobacterium tumefaciens*.¹⁶ A similar biosynthetic response was observed for cell cultures of *Centaurea erythraea* (Asteraceae) when exposed to extracts of yeast and the chemical elicitor methyl jasmonate.¹⁷ Taken together, these interesting results support the hypothesis that xanthones belong to the chemical defense arsenal employed by *H. perforatum* to combat biological stress factors, including infection by pathogens. Isolated xanthones from *H. perforatum*, however, have rarely been tested for their growth-inhibitory activity against these pathogens.

With these results and observations in mind, the goal of the current study was to conduct bioassay-guided fractionation of the root extract of *Hypericum perforatum*, testing extracts using the direct overlay bioautographic assay against *Colletotrichum fragariae*, *C. gloeosporioides* and *C. acutatum*. An isolated pure compound was subsequently evaluated using a dose–response format in a microdilution broth assay against *C. fragariae*, *C. gloeosporioides*, *C. acutatum*, *Botrytis cinerea*, *Fusarium oxysporum*, *Phomopsis obscurans* and *P. viticola*. In addition, owing to the lack of knowledge regarding the potential for the underground parts of *H. perforatum* to be used in alternative medicine, fractions and isolated pure compounds were evaluated *in vitro* for anti-inflammatory activity through inhibition of cyclooxygenase (COX)-1- and COX-2-catalyzed prostaglandin E₂ (PGE₂), as well as 5-lipoxygenase (5-LOX)-catalyzed leukotriene B₄ (LTB₄) formation.

MATERIALS AND METHODS

General experimental procedures

Vacuum liquid chromatography (VLC) was performed on either fine-grade silica gel 60 (0.04–0.06 mm; Roth, Karlsruhe, Germany) or medium-grade silica gel 40 (0.06–0.2 mm; Merck, Darmstadt, Germany). Analytical thin-layer chromatography (TLC) was performed on silica gel 60 F₂₅₄ plates (0.2 mm thickness; Merck), separation with mixtures of hexane–ethyl acetate (EtOAc) (75 : 25, 85 : 15 and 90 : 10), dichloromethane (DCM)–methanol (MeOH) (90 : 10, 95 : 5) or toluene–EtOAc–formic acid (60 : 55 : 5), detection under UV light at 254 and 365 nm, then visualization by spraying with H₂SO₄ (10% solution v/v in 95% aqueous ethanol (EtOH)) and then vanillin (5% solution w/v in 95% aq. EtOH)

reagents, followed by heating at 150 °C for 45 s. Preparative TLC was performed on silica gel F₂₅₄ plates (2.0 mm thickness; Merck). Solid-phase extraction (SPE) was performed with Isolute cartridges (C₁₈, 2g packing with 6 mL volume; Biotage, Uppsala, Sweden), elution with H₂O/MeOH mixtures. Preparative high-performance liquid chromatography (HPLC) was performed on a Varian HPLC system (Varian Inc., Palo Alto, CA, USA) equipped with Dynamax Model SD-1 and Varian Model SD-1 gradient pumps, in conjunction with a model UV-1 absorbance detector, using a Hibar® C₁₈ column (10 µm, 25×250 mm; Merck) at a flow rate of 25 mL min⁻¹, with either H₂O–MeOH or H₂O–acetonitrile (ACN) mixtures, monitoring at 245 nm. Semi-preparative HPLC and analytical HPLC and detection of UV spectra (λ_{max} in nm) were performed on an Agilent 1100 Separations Module equipped with a photodiode array detector (Agilent Technologies, Santa Clara, CA, USA) and using a LichroSpher 100-C₁₈ column (10 µm, 25 × 250 mm; Varian) at a flow rate of 2 mL min⁻¹, and a Zorbax SB-C₁₈ column (3.5 µm, 2.1 × 150 mm; Agilent Technologies) at a flow rate of 250 µL min⁻¹, using H₂O–MeOH or H₂O–ACN gradients, respectively. HPLC–diode array detection/electrospray ionization (ESI)–MS (pos.) data were obtained on a Thermo Finnigan Surveyor LC instrument with Thermo Quest Surveyor diode array detector, autosampler and MS pump, and a Thermo Finnigan LCQXP mass detector equipped with an ESI source run using Xcalibur software (Fisher Scientific, Waltham, MA, USA). Mass spectra were detected and recorded (scan range = m/z 50–1000, transfer capillary temperature = 350 °C, spray voltage = 5.00 kV, sheath gas flow = 70 units). Polarimetry was performed on a 241-MC polarimeter (PerkinElmer, Waltham, MA, USA) in a 10 cm microcuvette. Fourier transform infrared spectroscopic data were acquired using a PerkinElmer spectrometer, model-spectrum BX series, in cm⁻¹. ¹H-, ¹³C-, and 2D-nuclear magnetic resonance (NMR) experiments (HSQC, HMBC, DQF-COSY) were performed with Varian UNITYINOVA-400 and -600 MHz spectrometers. Chemical shift values (δ) were reported in ppm relative to tetramethylsilane (TMS, δ = 0) as an internal standard and coupling constants (J values) are given in Hertz. Compounds 1–3 (Fig. 1) were dissolved in either dimethyl sulfoxide or methanol-d₄ (MeOD) and spectra were recorded at 25 °C. Experimental parameters were as published by Seebacher *et al.*¹⁸

Plant material

Plants of *Hypericum perforatum* L. ssp. *perforatum* were cultivated in a common garden at the Experimental Station for Specialized Crops (Versuchsstation für Spezialkulturen) in Wies, Austria, and collected while in the phenological stage of full flower/early fruit (July). Botanical identity of the plant material was verified by S Crockett. A voucher specimen was deposited at the Institute for Pharmaceutical Sciences, Karl-Franzens-University in Graz, Austria. The roots were dried to a moisture content of less than 20 g water kg⁻¹ plant material prior to extraction.

Sample extraction and isolation

Hydrophobic extract—Root material (1.4 kg) was ground and extracted in a maceration tank with CH₂Cl₂ (4 L, 5 days), followed by MeOH (4 L, 5 days). In subsequent fractionation steps, further purification was pursued on the basis of TLC and HPLC characteristics, as well as the results from anti-inflammatory and antifungal bioassays. Combined CH₂Cl₂ extracts were evaporated *in vacuo* to give 23.2 g extract (16.6 g kg⁻¹ yield from dry roots). Of this extract, 17 g was subjected to VLC using fine-grade silica (200 g), eluting with a hexane–EtOAc gradient (5% steps, 700 mL per step, 25 mL fractions), followed by a final elution with 100% methanol (700 mL). TLC examination allowed the combination of similar fractions and yielded 12 fractions. On the basis of interesting bioactivity in fractions 9–12 (most potent in fraction 10), as well as TLC and HPLC profiles, fraction 10 (430 mg) was dissolved in MeOH and chromatographed over a C₁₈ SPE column, pre-conditioned with MeOH, and eluted with a MeOH–H₂O gradient (5 : 95 to 100 : 0). The

fractions eluting with 50–55% MeOH were further purified by preparative reversed-phase HPLC (gradient ACN–H₂O, 50 : 50 to 100 : 0 over 30 min, 100 : 0 for 10 min), resulting in the isolation of compound **1** (20.0 mg, 1.2 g kg⁻¹ CHCl₃ extract) as a yellow crystalline substance.

Hydrophilic extract—The combined MeOH extracts from the original maceration were evaporated *in vacuo* to give 37 g extract (26.4 g kg⁻¹ yield from dry roots). This concentrated extract was mixed with distilled water (900 mL) and partitioned against *n*-butanol (*n*-BuOH; 3×, 900 mL). The combined *n*-BuOH partitions were mixed 1 : 1 with fresh distilled water and concentrated *in vacuo* to give 3.4 g extract (2.4 g kg⁻¹ yield from dry roots). The aqueous phase was freeze-dried to give ~33 g extract (23.6 g kg⁻¹ yield from dry roots) which according to ¹H NMR consisted mainly of carbohydrates was not further investigated. The *n*-BuOH extract was subjected to VLC using medium-grade silica (40 g), eluting with a DCM–MeOH gradient (10% steps, 125 mL per step, 25 mL fractions), followed by a final elution with 100% MeOH (700 mL), which after combination according to similarities assessed with TLC and HPLC gave 15 fractions. Fraction 7 (714 mg) was dissolved in MeOH and chromatographed over a C₁₈ SPE column, pre-conditioned with MeOH, and eluted with an MeOH–H₂O gradient (10 : 90 to 100 : 0), with a final elution with DCM, yielding 11 subfractions. Subfraction 6 (31.3 mg) was subjected to preparative HPLC (isocratic, 70 : 30 ACN–H₂O for 15 min), resulting in the isolation of compound **2** (1.3 mg, 0.4 g kg⁻¹ *n*-BuOH extract). Subfraction 7 (56.5 mg) was subjected to preparative HPLC (gradient elution with ACN–H₂O, 35 : 65 to 100 : 0 in 30 min), resulting in the isolation of compounds **3** (3.0 mg, 0.9 g kg⁻¹ *n*-BuOH extract) and **1** (1.9 mg, 0.6 g kg⁻¹ *n*-BuOH extract). Subfraction 9 (84.8 mg) was dissolved in DCM and applied to a preparative TLC plate, which was developed with toluene–EtOAc–formic acid (60 : 55 : 5). Silica gel impregnated with a band detectable with 254 and 366 nm was scraped off, the substance eluted with MeOH, and further purified using preparative HPLC (gradient elution with MeOH : H₂O, 85 : 15 to 100 : 0 in 20 min), resulting in the isolation of compound **1** (1.0 mg, 0.3 g kg⁻¹ *n*-BuOH extract). Fraction 8 (424.5 mg) was dissolved in MeOH and chromatographed over a C₁₈ SPE column, conditioned with MeOH and eluted with an MeOH–H₂O gradient (10 : 90 to 100 : 0), with a final elution with DCM, yielding 11 subfractions. Subfraction 8 (32.9 mg) was subjected to semi-preparative HPLC (isocratic, 30 : 70 MeOH–H₂O for 15 min), resulting in the isolation of compound **1** (0.8 mg, 0.2 g kg⁻¹ *n*-BuOH extract).

Bioassay testing

In vitro assays against COX-1 and -2 enzymatic inhibition activity—Assays were performed in a 96-well plate format with purified prostaglandin H synthase (PGHS)-1 from ram seminal vesicles for COX-1 and purified PGHS-2 from sheep placental cotyledons for COX-2 (both Cayman Chemical Co., Ann Arbor, MI, USA) as previously described.^{19,20} The concentration of PGE₂, the main arachidonic acid metabolite in this reaction, was determined by a competitive PGE₂ enzyme immunoassay (EIA) kit (Assay Designs Inc., Ann Arbor, MI, USA). Indomethacin (ICN Biomedicals Inc., Aurora, OH, USA; COX-1 IC₅₀ = 0.9 μmol L⁻¹) and NS-398 (Cayman Chemical Co.; COX-2 IC₅₀ = 2.6 μmol L⁻¹) were used as positive controls.

In vitro assay for leukotriene formation inhibitory activity—The bioassay for inhibition of leukotriene formation was carried out in 96-well plate format as described by Adams *et al.* with slight modifications.²¹ Briefly, polymorphonuclear leukocytes with 5-LOX activity were isolated from venous human blood based on sedimentation rates and lysis tolerance. Cell viability was checked with 0.4% trypan blue solution. The cell suspension (4500 cells mL⁻¹) was incubated with the sample, CaCl₂, calcimycin A23187 and

arachidonic acid in a shaking water bath at 37 °C. After 10 min, incubation was stopped by addition of 10% HCO₂H. After centrifugation, the samples were diluted and the concentration of LTB₄ formed during incubation was determined by means of a competitive LTB₄ EIA kit (Assay Designs Inc.). Zileuton (Sequoia, Oxford, UK; IC₅₀ = 5.0 µmol L⁻¹) was used as positive control.

Direct bioautographic assay for activity against plant pathogenic fungi—Three extracts (DCM extract and *n*-BuOH and H₂O partitions from the MeOH extract) were initially evaluated for their antifungal activity against three important plant pathogenic fungi – *Colletotrichum* species – using direct bioautography. Pathogen production and the bioautographic procedures of Cantrell *et al.* were used to evaluate antifungal activity against fungal plant pathogens.²² Technical-grade commercial fungicides benomyl, cyprodinil, azoxystrobin and captan (Chem Service, Inc., West Chester, PA, USA) were used as fungicide standards at 2 mmol L⁻¹ in 2 µL of 95% ethanol. The DCM extract demonstrated non-selective activity against all three *Colletotrichum* species at 160 µg and 320 µg on TLC plates. Bioassay-guided fractionation was used to sequentially purify the antifungal compounds against *Colletotrichum* species. Conidia of *C. fragariae*, *C. acutatum* and *C. gloeosporioides* suspensions were adjusted to 3.0 × 10⁵ conidia mL⁻¹ with liquid potato-dextrose broth (PDB; Difco, Detroit, MI, USA) and 0.1% Tween-80. Using a 50 mL chromatographic sprayer, each glass silica gel TLC plate with fluorescent indicator (250 mm, Silica Gel GF Uniplate; Analtech, Inc., Newark, DE, USA) was sprayed lightly (until damp) three times with the conidial suspension. Inoculated plates were placed in a 30 × 13 × 7.5 cm moisture chamber (398-C; Pioneer Plastics, Inc., Dixon, KY, USA) and incubated in a growth chamber at 24 ± 1 °C with 12 h photoperiod under 60 ± 5 µmol m⁻² s⁻¹ light. Inhibition of fungal growth was measured 4 days after treatment. Sensitivity of each fungal species to each test compound was determined by comparing size of inhibitory zones. Fungal growth inhibition means for extracts and pure compounds were analyzed separately by ANOVA using SAS software, Version 8. Mean separations were performed based on Fisher's protected least significant difference (LSD) (*P* = 0.05). Statistical comparisons were made for fungal growth across compounds and of compounds across fungal growth. Means and standard deviations of inhibitory zone size were used to evaluate antifungal activity of solvent fractions and pure compounds (Table 1).

Microdilution broth assay—A standardized 96-well microdilution broth assay developed by Wedge and Kuhajek was used to evaluate antifungal activity towards *B. cinerea*, *C. acutatum*, *C. fragariae*, *C. gloeosporioides*, *P. viticola*, *P. obscurans* and *F. oxysporum*.²³ The fungicide captan was used as an internal fungicide standard in all assays. Each fungus was challenged in a dose–response format using test compounds, where the final treatment concentrations were 0.3, 3.0 and 30.0 µmol L⁻¹. Microtiter plates (Nunc MicroWell, untreated; Fisher Scientific, Roskilde, Denmark) were covered with a plastic lid and incubated in a growth chamber as described previously.²³ Sixteen wells containing broth and inoculum served as positive growth controls; eight wells containing solvent at the appropriate concentration and broth without inoculum were used as negative controls. The experiments were repeated three times. Mean absorbance values and standard errors were used to evaluate fungal growth at 48 h and 72 h, except for *P. obscurans* and *P. viticola*, for which the data were recorded at 144 h. Means for percent inhibition of each fungus at each dose of test compound relative to the untreated positive growth controls were used to evaluate fungal growth inhibition. The SAS system ANOVA procedure (Statistical Analysis System, Cary, NC, USA) was used to identify significant factors, and Fisher's protected LSD was used to separate means.²⁴ Fungal growth was then evaluated by measuring absorbance of each well at 620 nm using a microplate photometer (Packard Spectra Count; Packard Instrument Co., Downers Grove, IL, USA).

RESULTS AND DISCUSSION

Isolation of pure compounds

The dried ground roots of *H. perforatum* were extracted sequentially with DCM and MeOH. The MeOH extract was further partitioned between *n*-BuOH and H₂O. These three extracts, with the guidance of anti-inflammatory and antifungal bioassay results and TLC/HPLC profiling, were further fractionated using VLC, column chromatography, SPE, preparative and semi-preparative HPLC and preparative TLC. Three xanthones (Fig. 1) were yielded and identified as 1,6-dihydroxy-5-methoxy-4',5'-dihydro-4',4',5'-trimethylfurano-(2',3':3,4)-xanthone (**1**), 4,6-dihydroxy-2,3-dimethoxyxanthone (**2**) and *cis*-kielcorin (**3**).

The verification of the proposed structure for compound **1** (also known as 5-*O*-methyl-2-deprenylrheediaxanthone) was conducted through comparison of ¹H- and ¹³C-NMR and mass values with those cited in Rath *et al.*, in which publication it was first cited as a new natural compound isolated from the roots of *Hypericum roeperanum*.²⁵ This compound was also detected at low levels in the callus culture tissues of *H. perforatum* ssp. *angustifolium*.²⁶ Following a similar strategy for structural elucidation as used with **1**, the structure for compound **2** was proposed and verified through comparison of IR, UV, ¹H- and ¹³C-NMR and mass values with those cited in the literature.²⁷ Compound **2** has been previously reported from the aerial material of *Hypericum chinense*, but the current study represents the first report of **2** to our knowledge from *H. perforatum* ssp. *perforatum*.

The proposed structure of compound **3** was verified through comparison of data values published by Pinto *et al.*²⁸ Although the IR and mass spectra for *cis*-kielcorin (**3**) are similar to those of kielcorin, which has been reported from several *Hypericum* species including *H. perforatum*, the ¹H-NMR signal at δ_{H} 5.11 ($J = 8.4$ Hz) appears as a doublet, indicating an axial/equatorial coupling between the protons attached at C7' and C8' and a *cis* orientation of the benzodioxane moiety.^{29,30} Interestingly, compound **3** has generally been reported as a racemic mixture, as evidenced by the lack of sample optical activity.²⁸ During the course of this study, the optical activity of **1** was also recorded as zero, indicating that this isolated substance existed as a racemic mixture (**1a/b**). Compound **1** previously isolated from *H. roeperanum* displayed, however, a negative optical rotation of $[\alpha]^{20} - 15.4$ (Me₂CO; *c* 0.6), indicating that this compound was present in (or at the least, isolated from) the plant in only one form.²⁵ This observation suggests that specific biosynthetic enzymes, capable of producing a variety of isomeric xanthone derivatives, are present in *Hypericum* species. A more detailed examination of the distribution and presence of such compounds could provide valuable chemosystematic data, to be applied in combination with data from molecular systematic analyses for further elucidation of relationships among members of the genus.

Bioactivity of extracts and pure compounds

In the current study, the one-dimensional (1D) bioautography technique was used as a primary screening system to detect antifungal activity using *Colletotrichum* species and indicated that both DCM and MeOH extracts were active. During fractionation of these extracts, components present in DCM fractions 9–12 and *n*-BuOH fraction 7 obtained by VLC displayed promising antifungal activity against *C. gloeosporioides* and reduced activity against *C. acutatum* and *C. fragariae* (Table 1). Further fractionation of DCM fractions 9–12 and *n*-BuOH fraction 7 led to the repeated purification of **1** (total yield 21 mg (0.02 g kg⁻¹ yield dry roots), which showed only weak activity against the *Colletotrichum* species. We hypothesize that the initially observed activity was due to minor substance(s) in the original extracts, which owing to the low yield of subsequently isolated components (<0.5 mg, amounts insufficient for structural elucidation), could not be identified. It should be noted

that the total extract yield from the roots was quite low (43 g kg^{-1} dry roots), in part due to the high proportion of soluble carbohydrates, and only 33.89% of this extract could be further fractionated due to the insoluble nature (in DCM or MeOH) of the remaining components.

To examine whether compound **1** possessed antifungal activity against other plant pathogenic fungi of interest to our laboratory, **1** was subsequently evaluated using a 96-well microbioassay system against *C. acutatum*, *C. fragariae*, *C. gloeosporioides*, *B. cinerea*, *P. obscurans*, *P. viticola* and *F. oxysporum* (Fig. 2). The 96-well microdilution broth assay used an aqueous buffered broth culture system in a dose-response format to evaluate the activity of **1** in more detail. Secondary screening using this microbioassay system showed that **1** possessed its greatest activity at $30 \mu\text{mol L}^{-1}$ and inhibited the growth of *P. viticola* and *P. obscurans* by 96% and 47%, respectively, after 144 h exposure. Inhibition of *Colletotrichum*, *Botrytis* and *Fusarium* species growth was not observed.

The unexpected, but positive, result of further testing of **1** in the microbioassay system was the identification of this substance as one with significant activity against *P. viticola* and moderate activity against *P. obscurans*. Interms of bioactivity, **1** had previously been reported to display only a weak electron-transfer-based radical-scavenging activity, as assessed *in vitro* using 1,1-diphenyl-2-picrylhydrazyl (DPPH).³¹ The observed bioactivity against *P. viticola* and *P. obscurans* is of interest because the secondary metabolite class of xanthenes had not been previously identified among plant protectants with the potential to control *Phomopsis* species.

Only compound **1** was available in large enough amounts for testing in both antifungal and anti-inflammatory bioassays, although compounds **2** and **3** could also be evaluated in the latter bioassay. In initial bioassay testing against enzymes involved in inflammation response (COX-1, COX-2 and 5-LOX product formation), **1** demonstrated a very high ($98.46 \pm 0.59\%$) inhibition of LTB_4 formation in the *in vitro* assay, tested initially at $50 \mu\text{g mL}^{-1}$ ($146.1 \mu\text{mol L}^{-1}$). COX-1 was slightly ($18.41 \pm 6.50\%$) and COX-2 was moderately ($36.15 \pm 4.68\%$) inhibited at this concentration. Correspondingly, the *in vitro* activity against 5-LOX-mediated LTB_4 formation by **1** was evaluated in more detail and an $\text{IC}_{50} = 10.2 \mu\text{mol L}^{-1}$ was determined. Because 5-LOX inhibitors possess therapeutic potential against a variety of allergic and inflammatory conditions, in particular bronchial asthma, the ideal next step for this research would be to perform *in vivo* anti-inflammatory tests with **1** as well as with structurally related xanthenes.³² Norathyriol, a compound that has been identified in *Hypericum elegans*³³ and *H. umbellatum*,³⁴ and γ -mangostin³⁵ have been previously reported as xanthenes with pronounced 5-LOX inhibitory properties, showing the potential of this class of compounds as anti-inflammatory compounds.³⁶ Furthermore, xanthone dicarboxylic acids have been demonstrated to be potent LTB_4 receptor antagonists.³⁷

CONCLUSIONS

In this study, three xanthenes were isolated from the roots of *H. perforatum* and the bioactivity profiles for these compounds in *in vitro* antifungal and anti-inflammatory bioassays described. Although specific compounds with the potential to control the growth of *Collectotrichum* were not found, xanthone **1** was identified as a new inhibitor of the plant pathogenic fungi *Phomopsis obscurans* and *P. viticola* and of *in vitro* LTB_4 product formation. Conceição *et al.* identified the structurally related xanthenes mangiferin, 1,3,7-trihydroxy-6-methoxy-8-prenyxanthone and γ -mangostin in their control cell cultures of *H. perforatum*, and showed that the amounts of these compounds increased in cell cultures elicited with cell wall extracts of *C. gloeosporioides*.¹⁵ The *de novo* production of new xanthenes, tentatively identified as isomers of 1,3,6,7-tetrahydroxyxanthone-*C*-prenyl and

γ -mangostin (1,3,6,7-tetrahydroxyxanthone-*C*-bis-prenyl) was also observed, suggesting that these compounds may play a role in plant defense response. Interestingly, although mangiferin was cited as a major xanthone present in cell cultures of *H. perforatum* by Conceição *et al.*, the current study did not result in the isolation of this compound.¹⁵ At the time plant material used in this study was collected, the plants appeared lush and healthy, and neither symptoms of anthracnose disease nor herbivory by the leaf-feeding beetle *Chrysolina hyperici*, which can transmit *Colletotrichum* spores, were observed.¹² Particular questions that would be interesting to address in future research is whether particular biosynthetic pathways are favored under field as opposed to cell culture conditions; what baseline chemical diversity (particularly with respect to xanthone production) is present among *H. perforatum* plants from a diversity of populations of known genetic constitution; and the mode of action of selected xanthenes, both against enzymes involved in inflammation response and against *Phomopsis* species.

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Figure 1.
Xanthones isolated from the roots of *Hypericum perforatum*.

Figure 2. Growth inhibition of *Phomopsis viticola* (Pv) and *P. obscurans* (Po) after 144 h using 96-well microdilution broth assay in a dose response to **1** and the commercial fungicide standard captan.

Table 1
Antifungal activity results of *H. perforatum* extracts using direct bioautography with three *Colletotrichum* test species

Samples	Concentration	Mean fungal growth inhibition ^a (mm) ± SD					
		<i>C. acutatum</i>		<i>C. fragariae</i>		<i>C. gloeosporioides</i>	
		4 μ L	8 μ L	4 μ L	8 μ L	4 μ L	8 μ L
DCM extract, VLC fractions 9–12	20 ng mL ⁻¹	5.5 ± 0.71	6.0 ± 0.71	5.5 ± 0.71	6.5 ± 0.71	6.0 ± 0.71	8.5 ± 0.71
MeOH extract, VLC fraction 7	20 ng mL ⁻¹	4.5 ± 0.71	5.5 ± 0.71	4.0 ± 0.71	4.5 ± 0.71	5.5 ± 0.71	7.5 ± 0.71
Agrochemical standards	2 mmol L ⁻¹	2 μ L	–	2 μ L	–	2 μ L	–
Captan ^b	2 mmol L ⁻¹	11.71 ± 2.91	–	12.86 ± 2.17	–	12.38 ± 1.99	–
Azoxystobin ^b	2 mmol L ⁻¹	23.25 ± 3.05	–	23.0 ± 1.47	–	21.25 ± 2.38	–
DCM (blank control) only solvent	–	–	–	–	–	–	–
MeOH (blank control) only solvent	–	–	–	–	–	–	–

^aMean inhibitory zones and standard deviations (SD) were used to determine the level of antifungal activity against each fungal species.

^bTechnical-grade agrochemical fungicides (without formulation) with different modes of action were used as internal standards.